Digestion of biological materials using the microwave-assisted sample combustion technique

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Abstract

In this study a procedure for sample digestion based on sample combustion assisted by microwave radiation is proposed. Combustion is started by microwave radiation in the presence of oxygen under pressure using ammonium nitrate as aid for ignition. The system was adapted in a microwave oven with quartz closed vessels. A quartz piece is used simultaneously as a sample holder and as protection to the cap of quartz vessel from the flame generated in the combustion process. Sample was pressed into a pellet and placed on a disc paper in the holder and 50 μl of 50% m/v ammonium nitrate solution was added. The influence of the absorption solution (diluted and concentrated nitric acid or water) on the recoveries for Cu and Zn was evaluated. About 3 s of microwave irradiation was necessary to start the combustion. The combustion process was evaluated in relation to the influence of sample mass on the ignition time, combustion time and maximum operation pressure. Bovine liver, milk powder and oyster tissue certified reference materials were used to evaluate the accuracy of the procedure for determination of copper and zinc. Good agreement for zinc (96% to 103%) was obtained from bovine liver certified reference material when microwave combustion and microwave combustion followed by reflux were used to sample decomposition, even if water was used for absorption of analyte. For copper, the combustion followed by reflux of 5 min allows an agreement from 96% to 100%. Similar results were obtained for oyster tissue samples. However, for milk powder good agreement close to 100% was obtained only if 4 mol l$^{-1}$ HNO$_3$ was used with a reflux step. Results from the proposed procedure were also compared to those from conventionally used procedures for biological samples decomposition, such as wet digestion in open vessels and microwave-assisted digestion in closed vessels. The advantages of this procedure include the complete sample decomposition in less time than other procedures and the acid consumption was always lower than 2%. Another advantage is the low residual carbon content, less of 1.4% without reflux and less than 0.3% with the reflux step and the possibility of use of diluted acid as absorbing solution. Moreover, the new holder allows an effective protection of the vessel cap to burnt high masses.

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1. Introduction

Nowadays, the trace element content of biological samples is an important parameter for quality control due to the increasing use of these samples as food or dietary supplements. Bovine liver and milk powder are very spread commercial foodstuffs and the evaluation of the copper and zinc content in these samples must be evaluated in order to determine the dietary intake of these elements. In low concentration Cu and Zn are essential nutrients for humans and the recommended dietary allowance (RDA) for Zn is 8 to 11 mg/day and for copper is 0.9 mg/day [1,2]. If large amounts of zinc are ingested (more than 10 times the RDA) even for a short time, stomach cramps, nausea, and vomiting may occur [1]. For copper, the ingestion of high amounts can cause health problems such as Wilson’s disease that is a genetic disorder frequently associated with excessive copper exposure [1].

Nowadays, atomic absorption spectrometry is probably the most used technique for the determination of metals in several
samples [3]. Determinations by this technique can be usually performed by flame atomic absorption spectrometry (F AAS) when the concentration of the analyte is high enough as the case of Cu and Zn in biological samples. However, a previous sample decomposition step is frequently required in order to assure a suitable sample introduction and to minimize eventual interferences during the determination, mainly those related to the sample matrix composition.

Sample digestion techniques had a high improvement in the last decades mainly due to the development of microwave-assisted digestion systems, which combines high decomposition efficiency and low digestion time [4–6]. These characteristics, when combined with the use of closed vessels, allow the minimization of losses and contamination and nowadays the microwave-assisted decomposition in closed vessels can be considered as the state-of-art of sample digestion. However, in these systems concentrated acids are used, which may increase the blank value caused by elements present as contaminants. In addition, the presence of high acid concentrations in the digests may not be supported by some analytical techniques and a subsequent step to remove (or dilute) the acid excess may be necessary [7].

Combustion techniques had a spread use in the past, mainly due to the efficiency of sample oxidation and the high purity of oxygen when it is compared to the acids used in wet ashing procedures. Moreover, the oxidation process of organic matter is effective and may be performed in few minutes [8]. Different combustion systems have been proposed for trace element analysis, e.g. low temperature ashing [9], Wickbold combustion [10] and Trace-O-Mat system [11]. However, the more successful combustion techniques were those performed in closed vessels, e.g. oxygen flask combustion [12] and combustion bombs [13]. For these techniques the sample is burnt in the presence of oxygen excess and the combustion products are absorbed in a suitable solution into the same vessel used for combustion [8].

Oxygen flask combustion had their use limited mainly due to the unfavorable ratio of vessel surface to sample and also to the loss of some elements due to alloy formation. For combustion bombs the main drawback was related to the contamination from the metallic surfaces of the bomb. Moreover, these combustion techniques show low throughput that is an important figure for routine analysis.

Recently, a novel decomposition technique was proposed combining the advantages of classical combustion techniques with those from conventional closed systems heated by microwave radiation [14]. The so-called microwave-assisted sample combustion involves the combustion of organic samples in closed quartz vessels pressurized with oxygen and ignition being performed by microwave radiation. In this system, a small quartz holder, placed inside the quartz vessel, is the only change in relation to the conventional apparatus originally designed for conventional closed vessels wet digestion. In this procedure, a fast and complete combustion/digestion is performed in few seconds with minimum acid consumption and the only reagent used to decompose the sample is oxygen. In addition, this system allows a subsequent reflux step, if necessary, that is not available in other techniques as oxygen flask and combustion bombs. However, in the previous paper little information about the influence of absorbing solution concentration and time of reflux was described, as well as about the parameters related to combustion reaction.

In this study a microwave-assisted combustion procedure is proposed to decompose milk powder and bovine liver samples to further determine copper and zinc by F AAS. The performance of a commercial sample holder specially designed for the proposed procedure was evaluated and, contrary to the prototype holder previously proposed, this new holder allows the protection of the vessel cap from the flame generated during the combustion. The suitability of absorbing solutions was evaluated concerning their acid concentration and time to analyte absorption. Additionally, the need of a reflux step was also studied. The following parameters related to microwave-assisted sample combustion procedure (sample mass, maximum pressure during the combustion, kind of absorbing solution, use of an additional reflux step) were investigated.

2. Materials and methods

2.1. Instrumentation

A Multiwave 3000 microwave sample preparation system (Anton Paar, Graz, Austria) equipped with up to eight high-pressure quartz vessels (XQ-80, Cat. N° 18543) was used in this study. Vessels have an internal volume of 80 ml and the maximum operation pressure is 80 bar. Commercial quartz holders (Cat. N° 16427) were used to place the sample into the quartz vessel. Holders are made in quartz and their dimensions are shown in Fig. 1. The microwave irradiation program used for the combustion procedure was as follows: 1) 1400 W for 60 s,
2) 1400 W for 5 min (optional step for reflux), and 3) 0 W for 20 min for cooling if step 2 was applied. During steps 1 and 2, the cooling fan was selected at level 2 (level 3 was applied only for cooling). The pressure was monitored in each vessel by means of the original pressure sensors of the equipment. The software version was v1.27-SYNT, and the microwave program was previously modified to run with a maximum pressure rate of 3 bar s$^{-1}$. If the pressure rate was higher than 3 bar s$^{-1}$ the microwave irradiation is stopped and this time is considered as the ignition. In this work, each run was always performed with a minimum of four vessels.

Copper and Zn determinations were carried out with a Model Vario 6 FL atomic absorption spectrometer (Analytik Jena GmbH, Jena, Germany) equipped with a deuterium background corrector. Hollow cathode lamps (NARVA, G.L.E., Berlin, Germany) were used for Cu and Zn. The following parameters were selected: wavelength of 324.8 nm, spectral slit width of 0.8 nm, operated at 3 mA for copper and wavelength of 213.9 nm, spectral slit width of 0.5 nm, operated at 4 mA for zinc. A conventional burner (slit of 10 cm, ar + acetylene flame) was used throughout. The residual carbon (RC) was determined by inductively coupled plasma optical emission spectrometry (ICP-OES, Optima 2000, Perkin-Elmer, Norwalk, CT, USA), and measurements were made according to the conditions described in Ref. [15]. For the determination of the acid consumption for the proposed procedure an acid–base titration was performed using a digital potentiometer (Hanna, model pH211, Sarmeola di Rubano, Italy) equipped with a combined Ag/AgCl, a saturated KCl reference electrode, and a glass indicator electrode.

2.2. Samples and reagents

The following reference samples were used in this work: bovine liver (NIST SRM 1577), oyster tissue (NIST SRM 1566a) and milk powder (IRMM BCR 63), with certified Zn concentrations of 130±13, 830±57, and 49.0 μg g$^{-1}$, respectively. The corresponding Cu concentrations in the reference samples were 193±10, 66.3±4.3, and 0.602 μg g$^{-1}$. Milli-Q water (18.2 MΩ cm) was used to prepare all solutions. Analytical-grade reagents were used (Merck, Darmstadt, Germany). Concentrated nitric acid (65%) was distilled in a sub-boiling system (Milestone, model DuoPur). Working analytical solutions for Cu and Zn were prepared before use by serial dilution of stock reference solutions containing 1000 mg l$^{-1}$. Ammonium nitrate was dissolved in water, and that solution was used as igniter for the combustion procedure. A disc of filter paper (20 mm of diameter, 20 mg) with low ash content (Selecta, NR 589, Germany) was used to aid the combustion process. The paper was treated with a 10% (m/v) HNO3 solution for 20 min in an ultra sound bath and dried in an oven for 2 h at 60 °C before use.

2.3. Sample preparation

For the proposed combustion procedure, samples between 50 and 500 mg were pressed and weighed directly on the filter paper. After weighing, the samples were placed in the quartz holder. The quartz vessels were previously charged with 6 ml of absorbing solution (water, 2 mol l$^{-1}$ HNO3 and 14 mol l$^{-1}$ HNO3). The holder containing the sample was positioned into the quartz vessel, and 50 μl of ammonium nitrate solution was immediately added to the paper. After the closure of the vessels and capping of the rotor, they were pressurized with oxygen between 5 and 20 bar for 2 min. Vessels were pressurized using the device originally designed for pressure release when using it for conventional acid sample digestion. After the rotor with the vessels was placed inside the microwave cavity and the selected program for microwave radiation was started. After finishing the digestion, each vessel was carefully opened to release the pressure. The resultant solution was diluted with water and transferred into a 25 ml in a polypropylene vessel. Each run holders were soaked in concentrated HNO3 for 10 min followed by rinsing with water.

The decomposition by microwave-assisted digestion in closed vessels was carried out using the conditions recommended by the manufacturer (0.5 g of sample, 6 ml HNO3, 30 min at 1400 W). For the open vessels digestion the decomposition was performed using 0.5 g of sample and 8 ml of concentrated HNO3, heated at 130 °C by 3 h and 30 min in a heating block.

3. Results and discussions

3.1. Determination of the ignition and combustion times and maximum pressure in proposed procedure

A preliminary study was performed to evaluate some characteristics of the combustion process. These tests were made using bovine liver samples and pressure selected at 15 bar as initial oxygen pressure. The correspondent results are shown in Table 1. About 2 to 3 s of microwave irradiation were necessary to start the combustion. This time was not changed for all the investigated sample masses. For other biological materials studied in this work the ignition time was practically the same, which is in agreement to the previous results [14] which suggest that the combustion reaction occurs between the filter paper and ammonium nitrate. After the filter paper to be ignited the combustion is quickly extended to the sample. Therefore, in these conditions the combustion starts in a regular time and it is almost the same for different samples.

Concerning the combustion time it was observed, as expected, that with higher sample mass the combustion spends more time (Table 1). For 50 mg of sample only 7 s is necessary to

<table>
<thead>
<tr>
<th>Sample mass (mg)</th>
<th>Ignition time (s)</th>
<th>Time of combustion (s)</th>
<th>Maximum pressure (bar)</th>
<th>Maximum pressure increase (bar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>3</td>
<td>7</td>
<td>22.9±1.3</td>
<td>7.9</td>
</tr>
<tr>
<td>100</td>
<td>2</td>
<td>10</td>
<td>25.1±0.9</td>
<td>10.1</td>
</tr>
<tr>
<td>200</td>
<td>3</td>
<td>18</td>
<td>27.1±0.8</td>
<td>12.1</td>
</tr>
<tr>
<td>300</td>
<td>2</td>
<td>20</td>
<td>30.7±1.7</td>
<td>15.7</td>
</tr>
<tr>
<td>500</td>
<td>3</td>
<td>29</td>
<td>37.4±1.9</td>
<td>22.4</td>
</tr>
</tbody>
</table>
complete the combustion. With higher masses a correspondent increase of the combustion time is observed and about 30 s are necessary to burn 500 mg of bovine liver. This decomposition time is very short when compared to other procedures based on wet digestion techniques (in closed or open vessels).

The maximum pressure observed during the combustion was also influenced by the sample mass. Obviously, if a higher sample amount is combusted, its reaction with oxygen produces more gaseous compounds and the pressure is consequently higher. However, this study is important in view of safety reasons because the maximum operational pressure for the used quartz vessels is 80 bar. Then, it is necessary to assure that the pressure increase during the combustion is maintained at safe conditions. For 50 mg of sample an increase of about 8 bar was observed (initial oxygen pressure was 15 bar). For 300 mg of bovine liver the maximum pressure was about 31 bar. The maximum pressure observed to burn 500 mg of bovine liver is lower than 40 bar (which corresponds to a pressure increase of 22.4 bar). This maximum pressure attained is far from the maximum operational pressure recommended by the manufacturer. Then, it is necessary to assure that the pressure increase during the combustion is maintained at safe conditions. For all the tests it was observed a slight decrease of the pressure after the combustion related to the original oxygen pressure. It was supposed to be due to the consumption of oxygen and also to the formation and partial absorption of carbon dioxide by the solution inside the quartz vessel.

3.2. Influence of the absorbing solution and reflux time on Cu and Zn recoveries

The absorbing solution in combustion procedures has an important role on the quantitative recoveries of analytes, because the kind and the concentration of the solution are not the same for different analytes [13]. In Fig. 2 are shown the results for water, diluted (2 and 4 mol l\(^{-1}\) \(\text{HNO}_3\)) and concentrated nitric acid (14 mol l\(^{-1}\) \(\text{HNO}_3\)) used as absorbing solutions for Cu and Zn after combustion of bovine liver samples by the microwave-assisted combustion procedure.

For zinc, the agreement with the certified value for the reference sample was 96% using only water as absorbing solution without reflux. Using a 5 min for the reflux time the agreement was around 102%. If diluted or concentrated nitric acid solutions were used the agreements were between 99% and 103% with or without reflux. For this condition, the relative standard deviation (RSD) was reduced from 12% (water as absorbing solution) to less than 2%. This result is important because, for Zn, only diluted nitric acid (2 and 4 mol l\(^{-1}\)) is necessary to quantitative determinations and the risk of contamination due to the acid is then reduced. For copper, however, water cannot be used as absorbing solution and only 81% and 63% of agreement was observed using only water as absorbing solution with and without 5 min of reflux, respectively. For diluted nitric acid the correspondent agreements with the value of certified reference material were 97% and 84%. With the use of concentrated nitric acid as absorbing solution the agreements to the certified value were 103% and 97%, with and without 5 min of reflux, respectively. Thus, for zinc diluted nitric acid can be used even if a reflux step was not applied. In other hand, for copper, diluted nitric acid can be used only with a reflux step after the combustion.

Similar results were obtained for the determination of copper and zinc after the combustion of oyster tissue samples by the proposed procedure. Results are shown in Fig. 3. Using water as absorbing solution the agreements were 68% and 54% for Cu, and 95% and 80% for Zn, with and without 5 min of reflux, respectively. For both conditions, diluted and concentrated nitric acid, with and without a reflux step, the results for zinc were between 100% and 102% in relation to the certified value for this element. Then, for this element there is no need of use of concentrated nitric solution as absorbing solution that could minimize the risk of contamination due to this reagent.

![Fig. 2. Influence of absorbing solution and the reflux time on Cu and Zn determination in certified reference material of bovine liver samples after decomposition by the proposed procedure. All studies used 100 mg of sample, 50 μl of \(\text{NH}_4\text{HNO}_3\) (50% v/v), microwave power = 1400 W, 6 ml of absorbing solution, \(n \geq 3\).](image)
However, the agreement for copper using 2 or 4 mol l\(^{-1}\) nitric acid without reflux was only 93%, while results very close to 100% were obtained after a subsequent reflux step by 5 min. When concentrated nitric acid was used in both conditions, with and without reflux, the results were between 98% and 101% in agreement to the certified value for copper. Therefore, the reflux step has influence on the recoveries of analytes studied, and its application is necessary mainly for the subsequent determination of copper.

An important aspect that needs to be considered is that during the combustion the temperature of the absorbing solution does not exceed 80 °C. However, the temperature of the solution is quickly raised if a reflux step is applied for 5 min after the combustion step. During this time, the solvent boils and the vapors are condensed on the vessel walls and also on the quartz holder. This process allows an efficient interaction between the solvent and the quartz surfaces (in the condensed and vapor phases). Then, there is an efficient washing of the inner surfaces that facilitates complete recovery of the analyte to the absorbing solution.

For milk powder samples the results for Zn, using 2 mol l\(^{-1}\) HNO\(_3\) as absorbing solution, were close to 100% of agreement to the certified values only if a reflux step is applied. However, using 4 mol l\(^{-1}\) or concentrated HNO\(_3\) agreements very close to 100% were obtained without reflux. For Cu good agreements were obtained only using 4 mol l\(^{-1}\) or concentrated nitric acid with reflux. Then, for all the investigated samples a compromise was established related to the concentration of nitric acid the choice of the absorbing and the analyte. In this way, the solution of 4 mol l\(^{-1}\) HNO\(_3\) with a 5 min reflux was chosen for both analytes. However, it must take into account that the reflux time was kept at 5 min and no additional tests were performed to verify the effect of more time of microwave irradiation for the reflux step.

After the combustion process it was observed the presence of small white particles on the quartz holder, probably calcium oxides formed during the combustion due to reaction of the calcium present in these samples (about 1.3%) with oxygen. This residue was only dissolved completely if an acidic solution was used as absorbing/refluxing solution. Thus, the presence of particles can be related to the low recoveries when water was used. This fact was probably due to the association of the analyte with these particles.

### 3.3. Comparison of the microwave-assisted combustion procedure with other digestion procedures

Bovine liver samples were digested by four procedures as described in Table 2. For all the procedures the agreement to the certified value was very close to 100%. The only exception was the slight lower agreement for copper using the proposed procedure without reflux (about 96%). However, for zinc no reflux step was needed and only 30 s is necessary to digest the sample. The application of an additional reflux step in the microwave-assisted combustion procedure allows the determination for both analytes without significant changes in digestion time. This procedure allows a relatively high throughput in view of the microwaves used.

### Table 2

<table>
<thead>
<tr>
<th>Digestion procedure</th>
<th>Digestion time</th>
<th>Agreement (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cu</td>
</tr>
<tr>
<td>Combustion without reflux*</td>
<td>30 s</td>
<td>95.7±4.6</td>
</tr>
<tr>
<td>Combustion with reflux*</td>
<td>25 min</td>
<td>101.5±1.6</td>
</tr>
<tr>
<td>Microwave-assisted digestion (closed vessels)</td>
<td>50 min</td>
<td>98.8±4.1</td>
</tr>
<tr>
<td>Heating block (open vessels)</td>
<td>3 h and 30 min</td>
<td>104.2±2.4</td>
</tr>
</tbody>
</table>

* 4 mol l\(^{-1}\) HNO\(_3\) as absorbing solution.
of up to eight samples can be simultaneously processed in
25 min. In addition, the variation of results is reduced to 50% in
relation to other digestion procedures if an additional reflux step
is used. The results slightly higher than 100% observed to the
procedure using heating block were attributed to contamination
as this procedure was the only performed in open vessels.

The residual carbon content for bovine liver and milk powder
digests using the proposed procedure was about 0.3% and 1.4%
with and without reflux with 4 mol l\(^{-1}\) HNO\(_3\), respectively,
which confirm the high efficiency of decomposition of the
procedure. It must take into account that the residual carbon was
significantly higher by using other procedures: between 10% and
16% and between 2% and 5% using the digestion procedure
in open vessels and in microwave-assisted closed vessels pro-
cedures, respectively. In other hand, the acid consumption was
also evaluated and about 98% of the original acid remains after
decomposition which shows that the oxidation of sample occurs
mainly during the combustion process.

4. Conclusions

In general, the microwave-assisted combustion procedure for
biological materials showed a good performance due to the high
efficiency of organic matrix oxidation, relatively high through-
put and low final acidity of digests, which allows the mini-
mization of acid interferences and the residues generated. In
addition, the procedure is practically the same for different
samples that is suitable for the use in routine analysis. In relation
to safety aspects, even relatively high sample masses (up to
500 mg) can be burnt with a maximum pressure of 55 bar, that is
far from the maximum operation limit of 80 bar.

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References

[1] Public Health Service Agency for Toxic Substances and Disease Registry,
Department of Health and Human Services, Toxicological Profile for Zinc,
[2] Public Health Service Agency for Toxic Substances and Disease Registry,
Department of Health and Human Services, Toxicological Profile for
[4] Z. Mester, R. Sturgeon, Sample Preparation for Trace Element Analysis,
715–720.